pBC1 Milk Expression Vector Kit

For the Expression of Recombinant Proteins in the Milk of Transgenic Mice

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User Manual

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Important Information

Shipping/Storage

Shipping:

The pBC1 Milk Expression Vector Kit is shipped at room temperature.

Storage: Upon receipt--

- Store the pBC1 vector at -20°C.
- Store the Easy-DNA™ Kit at room temperature. For long-term storage (> 6 months), remove the mussel glycogen, RNase, and Protein Degrader and store at -20°C.

Kit Contents

The pBC1 Milk Expression Vector Kit contains the following reagents:

Reagent	Amount	Comments
pBC1 Vector	20 µg, lyophilized in TE, pH 8.0	Vector that expresses your gene of interest in the milk of transgenic mice
Easy-DNA™ Kit (see below for details)	150 reactions	Preparation of genomic DNA from mouse tails

Easy-DNA™ Kit

The Easy-DNA™ Kit included with the pBC1 Milk Expression Vector Kit contains the reagents listed below. Sufficient reagents are provided to isolate genomic DNA from 150 mouse tails. Store the Easy-DNA™ Kit at room temperature. For long-term storage (> 6 months), remove the mussel glycogen, RNase, and Protein Degrader and store at -20°C.

Item	Concentration	Amount Supplied
Solution A (Lysis Solution)	Proprietary	55 ml
Solution B (Precipitation Solution)	Proprietary	25 ml
TE Buffer	10 mM Tris-Cl, pH 7.5 1 mM EDTA, pH 8.0	100 ml
Mussel Glycogen	2 mg/ml in sterile water	750 µl
RNase	2 mg/ml in sterile water	750 µl
Protein Degrader	5 mg/ml in sterile water	750 µl

Additional Reagents

Additional Easy-DNA™ Kits are available separately from Invitrogen. Ordering information is provided below.

Item	Amount	Catalog no.
Easy-DNA™ Kit	150 reactions (if isolating genomic DNA from mouse tails)	K1800-01

Introduction

Overview

Introduction

The pBC1 vector is a 21.6 kb vector designed to facilitate expression of recombinant proteins in the milk of transgenic animals. The pBC1 Milk Expression Vector Kit is intended for use in performing feasibility studies in mice, with the expectation that the user is interested in eventual large scale recombinant protein production using larger animals. Successful expression of recombinant protein in transgenic mice has generally been indicative of successful expression in larger animals such as goats or cows (Young *et al.*, 1997). For feasibility studies, transgenic mice provide the added advantage of shorter generation times and faster evaluation than larger herd animals.

The pBC1 Milk Expression Vector Kit is specifically designed to:

- Provide instructions for cloning your gene of interest into the pBC1 expression vector
- Allow easy screening and identification of transgenic mice using the Easy-DNA™ Kit
- Provide general guidelines on the milking of transgenic mice and initial evaluation of recombinant protein expression in the milk

See below for information on generation of transgenic mice and scale up to larger animals.

This manual provides general guidelines for cloning your gene of interest into the pBC1 vector and instructions for identifying transgenic founder mice. **The manual is not intended to be an in-depth resource for the generation of transgenic mice. For detailed information and technical support on the generation and care of transgenic mice, we recommend collaboration with an experienced transgenic facility.**

Important

Please note that once successful recombinant protein expression has been performed in mice, scale up into larger animals will require the user to enter into a commercial agreement with Genzyme Transgenics Corporation (GTC) as described in the **Purchaser Notification** (see page 35). For more information, please contact GTC (see page 35).

Advantages of Recombinant Protein Production in Transgenic Milk

Transgenic animals are capable of producing biologically active recombinant proteins at high levels. In the pBC1 Milk Expression System, recombinant proteins are secreted at high levels into the milk of transgenic animals. Use of the pBC1 vector for recombinant protein production has resulted in yields as high as 35 g/L of recombinant protein in the milk of transgenic mice and 20 g/L in the milk of transgenic goats (Young *et al.*, 1997; Ziomek, 1998). Use of transgenic milk systems to express recombinant proteins offer a number of advantages over the use of cell culture systems:

- Milk provides a safe, abundant, and easily obtainable source of raw material for purification of expressed recombinant protein
- Yields of recombinant protein can be 10- to 1000-fold higher than cell culture systems (see the next page for more information)
- Transgenic lines maintain consistent protein expression across generations
- Posttranslational modifications of recombinant protein remain consistent, whereas posttranslational modifications in cell culture can vary depending on exact culture conditions (see page 3 for more information)

For a detailed review, refer to *Gene Expression Systems*, Chapter 14 (Meade *et al.*, 1999).

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Overview, continued

Recombinant Proteins Produced Using the pBC1 Vector

A broad range of recombinant peptides and proteins have been expressed in the pBC1 system, including orally active drugs such as glutamic acid decarboxylase and parenteral drugs such as antithrombin III. In functional studies of transgenically produced antithrombin III, recombinantly-produced protein was found to have a specific activity equal to that of plasma-derived protein (Edmunds *et al.*, 1998). A more detailed list of some of the recombinant proteins that have been expressed using the pBC1 vector is provided in the **Appendix** (see page 29). For more information and references on recombinant protein production and yields in larger herd animals, please refer to the Genzyme Transgenics Corporation Web site at:

www.genzyme.com/transgenics

β-Casein Promoter

The pBC1 vector uses the goat β-casein promoter to drive high-level expression of the recombinant protein of interest. The goat β-casein promoter is a tissue-specific promoter that targets expression of the gene of interest almost exclusively to the lactating mammary gland (see below), with some minor expression in skeletal muscle and skin (Roberts *et al.*, 1992). Although the pBC1 vector is primarily intended for high-level recombinant protein production in the milk of transgenic mice, the specificity of the promoter also allows study of the effects of a protein of interest on a specific target tissue (e.g. the mammary tissue).

The Mammary Gland and Characterization of Milk

The synthesis of milk is carried out by mammary epithelial cells in the mammary gland. These cells are also responsible for all posttranslational modifications including glycosylation and phosphorylation. Typically, a mammary gland can synthesize and secrete approximately 2 grams of milk per gram of tissue per day (Young *et al.*, 1997). The mammary gland is a natural bioreactor with cell densities up to 100- to 1000-fold greater than most cell culture systems. This cell density translates to approximately 2×10^8 cells per gram of tissue, with a milk output of approximately 10^{-8} grams per cell per day.

Milk is a well-characterized colloidal mixture of fats and proteins, and is composed of the major proteins listed below. Further information about milk proteins may be found in published reviews (Maga and Murray, 1995; Young *et al.*, 1997).

Milk Protein	Percentage of Total Protein
Casein (α_{S1}, α_{S2}, β, κ)	80
β-lactoglobulin	10
α-lactalbumin	4
Enzymes, plasma proteins	3
Immunoglobulins	2
Albumin	1

Recombinant proteins that are produced in transgenic animals are designed to be secreted into the milk along with these other milk proteins and components. Large-scale purification of the recombinant protein of interest from milk typically involves clarification as the first step to remove fats. In the case of feasibility studies in mice, the milk is simply diluted and loaded onto an SDS-polyacrylamide gel for detection of the recombinant protein of interest by Coomassie blue staining or by Western blot.

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Overview, continued

Posttranslational Modification of Recombinant Proteins in Transgenic Animals

Transgenic animals are capable of producing complex human recombinant proteins that are glycosylated and phosphorylated (Denman *et al.*, 1991). Specific glycosylation enzymes vary somewhat by species, therefore, transgenically-produced protein may vary from purified human-derived protein. Within a single transgenic line, however, post-translational modification is consistent among animals and across generations. In the case of transgenically produced human antithrombin III, biological activity was similar to that of plasma-derived antithrombin III, although differences did exist in glycosylation patterns (Edmunds *et al.*, 1998).

Experimental Outline

The table below describes the basic steps needed to clone your gene of interest into the pBC1 vector and to express your recombinant protein in transgenic mouse milk. For more details, please refer to the pages indicated.

Step	Action	Page(s)
1	Develop a cloning strategy to ligate your gene of interest into the pBC1 vector.	6-11
2	Ligate your gene into the desired vector and transform into a *rec*A, *end*A, *E. coli* strain (e.g. TOP10). Select transformants on LB agar plates containing 50 to 100 μg/ml ampicillin.	12
3	Use colony PCR or colony hybridization to screen for transformants that contain the insert of interest.	12-13, 30
4	Isolate plasmid DNA and sequence your pBC1 construct to confirm that your insert is cloned in the proper orientation and contains the appropriate features required for expression.	14
5	Perform restriction digestion, agarose gel electrophoresis, and electroelution to remove the prokaryotic sequences and isolate the transgene construct.	16-17
6	Prepare transgene DNA for microinjection.	15-18
7	Send DNA to transgenic core facility or other commercial transgenic facility to generate transgenic mice.	19
8	Screen mice to identify transgenic founders.	20-23
9	Breed female transgenic founder mice. Proceed to step 11.	24
10	Mate male transgenic founder mice and screen the resulting progeny to identify female transgenic mice (F1 generation). Breed F1 female transgenic mice.	24
11	Harvest milk from female transgenic mice once they have produced litters.	24-26
12	Assay the milk for recombinant protein expression.	27

Methods

General Cloning Information

Introduction

The following section provides general information and guidelines for maintaining and propagating the pBC1 vector.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Important

The pBC1 vector is designed to help you express a gene of interest in the milk of transgenic mice as a means of evaluating the feasibility of proceeding towards large-scale recombinant protein expression in larger transgenic animals. Although the vector has been engineered to help you express your protein of interest in transgenic mice in the simplest, most direct fashion, use of the system is geared towards those users who possess a sophisticated knowledge of molecular biology techniques. We highly recommend that users be familiar with the principles of transgenesis, the care and handling of mice, and protein purification techniques.

For molecular biology protocols and information about manipulating and handling mice, please refer to the following general reference sources:

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1994). *Current Protocols in Molecular Biology* (New York: Greene Publishing Associates and Wiley-Interscience).

Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994). *Manipulating the Mouse Embryo*, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

The pBC1 vector is over 21 kb in size. Because of its large size, extra care should be exercised when handling and propagating the vector to avoid shearing the DNA or losing vector sequences. Pay particular attention when performing manipulation steps including cloning, transformation, and DNA preparation.

E. coli Host Strain

Many *E. coli* strains are suitable for the propagation of the pBC1 vector including TOP10 (Catalog no. C610-00) or DH5α. We recommend that you propagate and maintain the pBC1 vector and your transgene construct in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A). To facilitate the uptake of large plasmids such as pBC1, we also recommend that you use an *E. coli* strain that is. For your convenience, TOP10 is available as electrocompetent or chemically competent cells from Invitrogen.

Item	Quantity	Catalog no.
Electrocomp™ TOP10	5 x 80 µl	C664-55
One Shot™ TOP10 (chemically competent cells)	21 x 50 µl	C4040-03

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General Cloning Information, continued

E. coli Transformation

You may use any method of choice for transformation. Electroporation is the most efficient and the method of choice for large plasmids such as pBC1. Chemical transformation is the most convenient for many researchers and is also suitable.

Maintenance of pBC1 Vector

To propagate and maintain the pBC1 vector, resuspend the vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.

Use this stock solution to transform a *rec*A, *end*A, *E. coli* strain like TOP10, DH5α, or equivalent. Select transformants on LB agar plates containing 50 to 100 µg/ml ampicillin. Be sure to prepare a glycerol stock of each strain containing plasmid for long-term storage.

To prepare a glycerol stock:

1. Streak the original colony out on an LB agar plate containing 50 µg/ml ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Designing Transgenes

Introduction

This section contains guidelines for designing your transgene construct. There are many factors to consider when designing a transgene including the size of the DNA construct, inclusion of introns and exons, use of genomic sequences, propagation and maintenance of the vector construct, and detection of the transgene in mice. A brief discussion of each of these factors is provided below. For more details, please refer to Hogan *et al.*, 1994.

Size of the DNA Construct

Transgenic mice have been successfully generated from microinjection of DNA fragments as large as 70 kb (Strouboulis *et al.*, 1992). In general, the size of the transgene does not appear to affect the frequency of obtaining transgenic mice, but is limited more by cloning and handling considerations of the vector construct itself. For pBC1-derived DNA fragments ranging from 25-35 kb in size, the success rate of obtaining transgenic mice is approximately 10% of the total mice obtained (Genzyme Transgenics Corporation, unpublished observations).

To facilitate propagation and maintenance of such large transgene constructs, the pBC1 vector contains cosmid packaging sequences that allow packaging of the vector construct of interest into cosmids should the size be too large for efficient conventional bacterial transformation and propagation.

Structure of the Gene of Interest

Transgenic mice have been successfully generated from constructs in which the gene of interest is expressed from either a cDNA or a genomic fragment. However, many studies have shown that the levels of gene expression obtained with genomic DNA-based constructs are generally higher than those obtained with cDNA-based constructs (Brinster *et al.*, 1988).

Inclusion of Introns

Inclusion of introns in the transgene construct has been shown to increase the levels of transgene expression dramatically (Brinster *et al.*, 1988). The mechanism for the increased expression of certain transgenes is not entirely known. In some cases, the increased expression associated with a particular transgene can be attributed to the presence of regulatory sequences within the intron sequences.

Note

The introns and exons that are included in the transgene construct need not necessarily be derived from the gene of interest as expression from transgenes can be substantially enhanced by the inclusion of heterologous introns and exons in the construct (Choi *et al.*, 1991; Palmiter *et al.*, 1991). In the pBC1 vector, genomic sequences from the goat β-casein gene flank the cloning site for your gene of interest. The presence of β-casein genomic sequences (introns and exons) allows you to clone your gene of interest into pBC1 as either a cDNA or a genomic fragment. Your gene of interest will be inserted in such a way that it lies between exons 2 and 7 of the goat β-casein gene (see page 10 for a map of the vector).

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Designing Transgenes, continued

Codon Usage

For optimal expression of your recombinant protein, the codon usage should be maximized for mammals (e.g. human, mouse, goat). If you are planning to express your protein of interest from a human cDNA or genomic fragment, minimal optimization for codon usage is necessary as codon preferences are generally similar within most mammalian species.

If you are expressing a protein of interest from a prokaryotic or yeast gene, we recommend that you translate the mRNA sequence of your gene to determine the codon usage. If your gene of interest contains codons that are not preferred for mammals, you may want to perform mutagenesis to optimize the codon usage for mammals.

For more information about codon usage, please refer to the Codon Usage Database on the World Wide Web at:

www.dna.affrc.go.jp/~nakamura/CUTG.html

Removal of Prokaryotic Sequences

Transgenic expression vectors generally contain prokaryotic sequences that allow selection and propagation of the vector in bacterial strains or in cosmids. The presence of prokaryotic sequences does not appear to affect the frequency of integration of the micro-injected transgene, but can severely inhibit the expression of the transgene in the animal (Chada *et al.*, 1985; Krumlauf *et al.*, 1985; Townes *et al.*, 1985). To circumvent this problem, most protocols for generating transgenic mice recommend the removal of prokaryotic sequences from the construct prior to introduction of the transgene construct into mice. The pBC1 vector contains prokaryotic sequences from nucleotides 15761-21628 (see vector map on page 10) that may be removed from the transgene construct by restriction digestion of the vector with the *Not* I and *Sal* I enzymes and separation of DNA fragments by agarose gel electrophoresis. Other restriction sites are available.

Screening for Potential Transgenic Mice

When designing your transgene construct, you should take into consideration the structural features of the transgene that will allow you to distinguish your gene of interest from a possible wild-type mouse homolog. When screening mice to identify transgenic founders, you will need to have a probe that can distinguish between the transgene and the native mouse gene. Generally, transgenes will integrate into the genome in head-to-tail arrays. Typically, mice are initially screened for the presence of the transgene by PCR. Putative transgenic mice are then analyzed for transgene integration and copy number by restriction digestion and Southern blot analysis. Optimally, you should design a probe that will allow you to identify transgenic mice as well as estimate the number of copies of the transgene that have integrated into the genome.

Types of Protein to Express

A variety of recombinant proteins have been successfully expressed in transgenic milk systems including human serum albumin, antithrombin III, and human long-acting tissue plasminogen activator (Denman *et al.*, 1991; Edmunds *et al.*, 1998; Young *et al.*, 1997). While many types of proteins can be expressed in the pBC1 Milk Expression System, proteins that are normally secreted tend to express at the highest levels in milk. In addition, it is important to note that certain proteins that are particularly toxic to mammalian tissues may also have dramatic effects on transgenic animal development.

Detection of Recombinant Protein

Please note that the pBC1 vector does **not** include an epitope tag for detection of recombinant protein. You will need to have an antibody to your recombinant protein of interest in order to detect expression by Western blot.

Cloning into pBC1

Introduction

General considerations for cloning your gene of interest into the pBC1 vector are described below. For a map and a description of the features of pBC1, please refer to pages 10-11.

Prokaryotic Sequences

The prokaryotic sequences in the pBC1 vector are derived from the pHC79 plasmid (Hohn and Collins, 1980) and include:

- the ampicillin resistance gene for selection in *E. coli*
- pBR322 origin of replication for maintenance and low-copy replication in *E. coli*
- cosmid packaging sequences that provide the user with the option of packaging larger constructs using lambda phage cosmid technology (see Sambrook *et al.*, 1989 for protocols)

Insulator Sequences

The pBC1 vector contains two tandem copies of a sequence located immediately upstream of the β-casein promoter that has been shown to function as a chromatin insulator (Chung *et al.*, 1997; Chung *et al.*, 1993). The insulator sequences were originally derived from the 5´ region of the chicken β-globin gene (Chung *et al.*, 1997; Chung *et al.*, 1993). When incorporated into transgene constructs, the insulator sequences have been shown to reduce the influence of cis-acting regulatory elements on the activity of the transgene (Talbot *et al.*, 1989). The presence of the insulators eliminates position effects caused by the integration of transgenes into specific sites in the mouse genome, and effectively reduces variability in the expression levels of transgenically-produced recombinant proteins (Talbot *et al.*, 1989).

Kozak Consensus Sequence

The goat β-casein exon sequences included in the pBC1 vector do not contain an ATG start codon, therefore, your insert should contain a Kozak translation initiation sequence **with** an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Please note that other sequences are possible (see references above), but the G or A at position -3 and the G at position +4 are the most critical (shown in bold). The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

Secretion Signal

In order for your protein of interest to be efficiently secreted into the milk of the transgenic mice, your construct **must** contain a secretion signal. If your gene of interest is a secreted protein, you may use the native secretion signal for your gene. If your protein does not have a secretion signal, then you must add a heterologous secretion signal to your construct. We recommend that you use the secretion signal for the goat β-casein gene (Persuy *et al.*, 1995; Roberts *et al.*, 1992) to allow secretion of your protein into the milk. The peptide sequence of the β-casein secretion signal is provided below:

MKVLILACLVALAIA-

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Cloning into pBC1, continued

Translation Termination and Polyadenylation Sequences

Your insert must contain a stop codon to allow termination of translation.

The pBC1 vector contains a large (7.1 kb) genomic fragment from the goat β-casein gene following the *Xho* I cloning site (see below). This 3´ genomic fragment contains β-casein exons and introns as well as the polyadenylation sequences necessary for efficient termination of transcription and polyadenylation of mRNA. If the insert for your gene of interest contains the polyadenylation signal or other regulatory sequences, you may replace the β-casein polyadenylation signal with the polyadenylation signal for your gene. We recommend that your insert also include 3´ genomic sequences (i.e. exons and introns) in addition to the polyadenylation signal. To remove the 7.1 kb 3´ β-casein fragment from pBC1:

1. Digest the pBC1 vector with *Xho* I and *Not* I restriction enzymes.
2. Separate the 3´ β-casein fragment from the pBC1 vector by agarose gel electrophoresis and isolate the DNA fragment containing the remainder of the pBC1 vector.
3. Clone your gene of interest containing the polyadenylation signal into pBC1.

Cloning Site

The pBC1 vector contains a unique *Xho* I restriction site to allow cloning of your gene of interest downstream of the goat β-casein promoter. Heterologous exons and introns from the β-casein gene have been included such that your insert will be flanked by portions of exon 2 and 7 of the β-casein gene (see vector map on the next page). If you plan to PCR amplify your insert, you must design your primers such that the amplified fragment will contain ends compatible with *Xho* I. We recommend that you sequence PCR products prior to generation of transgenic animals in order to avoid sequence errors.

Note

Please note that your recombinant protein will not include any amino acids from the β-casein exons if you include an ATG start codon and a stop codon within your insert.

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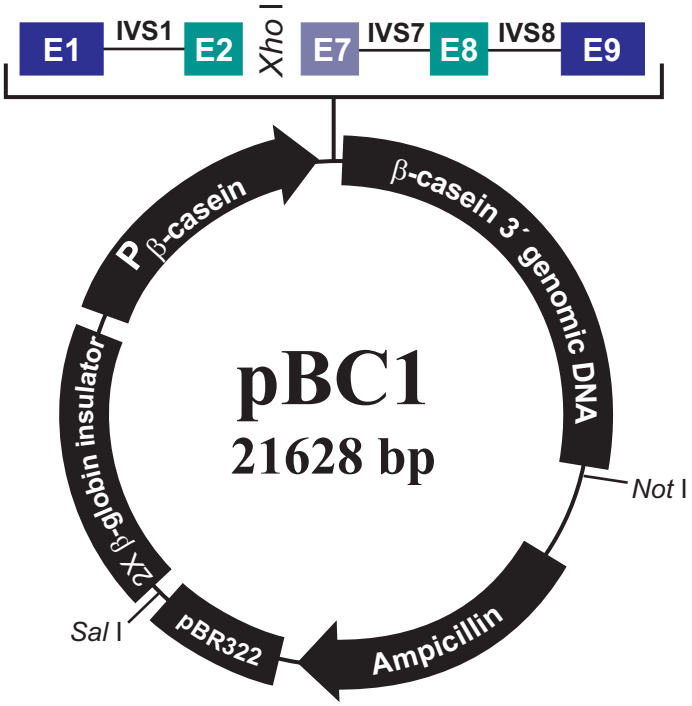
Cloning into pBC1, continued

Map of pBC1 Vector

The figure below summarizes the features of the pBC1 vector. Please note that although all β-casein exons are transcribed, they will be untranslated if you clone your insert into the *Xho* I site and include an ATG start codon and a stop codon within your insert. **The complete sequence for pBC1 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).** For more information about the goat β-casein genomic sequences, please refer to Roberts *et al.*, 1992.



Comments for pBC1: 21628 nucleotides

Chicken β-globin insulator (2X): bases 12-2412
Goat β-casein promoter: bases 2427-6527
TATA box: bases 6499-6506
β-casein exon 1 (untranslated): bases 6528-7575
β-casein intron 1 (IVS1): bases 6576-8596
β-casein exon 2 (partial, untranslated): bases 8597-8602
Xho I cloning site: bases 8609-8614
β-casein exon 7 (partial, untranslated): bases 8620-8637
β-casein intron 7 (IVS7): bases 8638-9224
β-casein exon 8 (untranslated): bases 9225-9266
β-casein intron 8 (IVS8): bases 9267-9993
β-casein exon 9 (untranslated): bases 9994-10245
β-casein 3´ genomic fragment: bases 10246-15760
pHC79 cosmid vector sequences: bases 15761-21628 (complementary strand)
bla promoter: bases 15869-15967
Ampicillin (*bla*) resistance gene: bases 15968-16828
pBR322-derived origin: bases 16972-17645

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Cloning into pBC1, continued

Features of the pBC1 Vector

The table below summarizes the features of the pBC1 vector (21628 bp). All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
Chicken β-globin insulator (2X)	Shields the β-casein promoter from the influence of nearby regulatory elements and allows position-independent expression of the gene of interest in transgenic mice (Chung *et al.*, 1997; Chung *et al.*, 1993)
Goat β-casein promoter	Permits inducible expression of your recombinant protein in the mammary epithelial cells of transgenic mice (Persuy *et al.*, 1992; Roberts *et al.*, 1992; Young *et al.*, 1997)
Goat β-casein gene (3.7 kb genomic fragment includes exon 1, parts of exons 2 and 7, exon 8, and exon 9)	Structural sequences that enhance expression of your recombinant protein in mammary epithelial cells (Roberts *et al.*, 1992; Young *et al.*, 1997)
Xho I cloning site	Allows insertion of your gene between exons 2 and 7 of the goat β-casein gene
3´ untranslated region (UTR) of goat β-casein gene	Permits translation termination and polyadenylation of mRNA (Roberts *et al.*, 1992)
pHC79 cosmid vector sequences	Contains prokaryotic sequences that allow selection and propagation of the pBC1 vector in *E. coli*, and includes cosmid packaging sites to allow packaging of constructs in lambda phage (Hohn and Collins, 1980)
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene in *E. coli*
Ampicillin (*bla*) resistance gene	Selection of transformants in *E. coli*
pBR322-derived origin	Maintenance and low copy replication in *E. coli*

Transformation and Screening

Introduction

Once you have ligated your gene of interest into pBC1, follow the guidelines below to transform and screen your clones. For detailed protocols, please refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) and *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989).

E. coli Transformation

Prepare competent *rec*A, *end*A, *E. coli* cells (e.g. TOP10) using your method of choice. For efficient transformation of pBC1, we recommend using electroporation to transform your ligation mixtures. Transform your ligation mixtures and select on LB agar plates containing 50 to 100 µg/ml ampicillin. For fast and easy microwaveable preparation of Low-Salt LB plates containing ampicillin, imMedia™ Amp Agar (Catalog no. Q601-20) is available from Invitrogen. Please call Technical Service (see page 33) for more information.

Note

If the efficiency of your *E. coli* transformation is low, you may want to use lambda phage cosmid technology to package your pBC1 construct as a cosmid. For more details and protocols, please refer to Molecular Cloning: A Laboratory Manual (Sambrook *et al.*, 1989).

Screening Transformants

Using a large vector such as pBC1 for cloning requires the screening of large numbers of *E. coli* transformants for the presence of the insert of interest. We recommend large scale screening of at least 100-200 transformants by colony PCR. Alternatively, performing filter lifts and colony hybridization are also an effective means of screening large numbers of transformants. A sample protocol for screening transformants by colony PCR is provided on the next page for your convenience. A sample protocol for colony hybridization is provided in the **Appendix**, page 30.

PCR Primers

To screen *E. coli* transformants by colony PCR, you will need to design PCR primers that will allow you to amplify your insert of interest as well as determine the orientation of your insert in the pBC1 vector. Optimally, the primers may also be used to sequence your insert after you have identified transformants. We have successfully used the following primers to screen and sequence *E. coli* transformants:

Primer	Sequence	Location (bp)
Forward	5´-GATTGACAAGTAATACGCTGTTTCCTC-3´	8554-8580
Reverse	5´-CATCAGAAGTTAAACAGCACAGTTAG-3´	8653-8678

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Colony PCR

A sample protocol for performing colony PCR is provided below. Other protocols are suitable. Please refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) for additional information.

1. Pick 100-200 colonies. For each colony, streak a small patch on a fresh LB agar plate containing 50 µg/ml ampicillin. Incubate the plate overnight at 37°C.
2. Prepare a PCR cocktail consisting of the components listed below. Use a 20 µl volume for each sample. Multiply by the number of colonies to be analyzed (e.g. 100-200). Aliquot 20 µl of the PCR cocktail into each microcentrifuge tube.

10X PCR Buffer	2 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl)	1 µl
Sterile Water	15.5 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	20 µl

3. Remove a small amount of each *E. coli* transformant from the LB plate with a toothpick and resuspend the *E. coli* in the microcentrifuge tube containing the 20 µl of PCR cocktail. Remember to label your tubes.
4. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
5. Amplify DNA using the following general cycling parameters or parameters of your choice. Please note that cycling parameters may vary depending on the size of your insert.

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	58°C	
Extension	1 minute	74°C	
Final Extension	7 minutes	74°C	1X

6. Visualize PCR products by agarose gel electrophoresis. **Note:** Bufferless, precast agarose E-Gels™ (Catalog no. G5000-01) are available from Invitrogen for fast and easy electrophoresis. Please see our Web site (www.invitrogen.com) or call Technical Service (see page 33) for more information.

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Transformation and Screening, continued

Once you have identified transformants containing pBC1 plasmid with inserts, we recommend that you sequence your construct to confirm that your gene of interest is cloned in the proper orientation in pBC1, and that it includes a secretion signal, an ATG initiation codon, and a stop codon. For sequencing, you may use the primers that were used to screen your *E. coli* transformants or any other appropriate primers.

Plasmid Preparation

For subcloning and analysis of transformants to identify those containing plasmids with inserts in the correct orientation, mini-prep quality plasmid DNA is sufficient for success.

For sequencing and preparative purposes, plasmid DNA of high purity is required. Plasmid DNA for sequencing and preparative purposes must be very clean and free from phenol and sodium chloride. We recommend isolating plasmid DNA using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or CsCl gradient centrifugation. The S.N.A.P.™ MidiPrep Kit is a medium-scale plasmid preparation kit that allows isolation of 10-200 µg of plasmid DNA from 10-100 ml (see **Note** below) of bacterial culture. Plasmid DNA purified using the S.N.A.P.™ MidiPrep Kit can be used directly to prepare DNA for microinjection.

Note: Since pBC1 is a low-copy number plasmid, you will need to increase the amount of bacterial culture that you use for plasmid purification. We recommend that you increase the volume of your bacterial culture 3 to 5-fold to obtain enough purified plasmid for further manipulations.

Preparation of DNA for Microinjection

Introduction

Once you have cloned your gene of interest into pBC1 and have prepared clean plasmid preparations of your construct, you are ready to prepare DNA for microinjection into fertilized eggs. A number of factors can affect the efficiency of gene transfer including:

- Using linear or circular DNA
- DNA concentration
- Purity of the DNA
- Composition of the microinjection buffer

Before preparing DNA for microinjection we recommend that you read through this section and discuss DNA preparation with your transgenic facility.

Linear DNA vs. Circular DNA

Transgenic mice have been obtained by microinjection of either linear DNA or supercoiled DNA into fertilized eggs. However, microinjection of linear DNA appears to increase the integration frequency of the injected transgene. Brinster *et al*., 1985 have found that injection of linear DNA can increase the integration frequency five-fold when compared to injection of supercoiled DNA. Most of the linear DNA molecules will integrate into the chromosome in a head-to-tail array. To increase your chances of obtaining transgenic mice, we recommend that you linearize your DNA prior to microinjection.

When designing a strategy to linearize your DNA for microinjection, remember to linearize your pBC1 construct in such a way that most (or all) of the prokaryotic sequences are removed from the DNA fragment to be microinjected. We recommend digesting the pBC1 vector with *Not* I and *Sal* I to remove the prokaryotic sequences (see vector map on page 10). Please note that these restriction sites may not be available if they are found in your gene of interest. Other restriction sites are possible.

DNA Concentration

In most cases, DNA for microinjection is prepared as a concentrated stock solution and then diluted prior to microinjection. On average, approximately 1-2 picoliters of DNA solution is injected into each fertilized egg. Although the total amount of DNA that is microinjected varies with each injection and is difficult to quantify, the concentration of the DNA solution can effect the integration frequency and the chances of obtaining transgenic mice. Generally, the optimal concentration of the diluted DNA solution for microinjection ranges from 1-10 μg/ml. The number of transgenic mice obtained decreases when the DNA concentration is less than 1 μg/ml, while embryo survival decreases dramatically when the DNA concentration is greater than 10 μg/ml.

Purity of the DNA for Microinjection

DNA for microinjection into fertilized eggs must be extremely clean and free of all contaminants (e.g. traces of phenol, ethanol, enzymes, or agarose) that might harm the egg and any particulate matter that could clog the injection needles. All solutions used to prepare DNA for microinjection should be filtered through a 0.22 μm filter (Millex-GV, Millipore, Catalog no. SLGV R25 LS). We recommend purifying your linearized DNA by agarose gel electrophoresis followed by electroelution, CsCl centrifugation, and extensive dialysis. Other standard methods for DNA preparation are suitable. A protocol for preparation of DNA for microinjection is provided for your convenience (see next page). For details, please refer to *Manipulating the Mouse Embryo* (Hogan *et al.*, 1994).

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Preparation of DNA for Microinjection, continued

Microinjection Buffer

The recipe for microinjection buffer used to resuspend your DNA construct can vary, but typically contains the following components:

- 5-10 mM Tris, pH 7.4
- 0.1-0.25 mM EDTA

Please note that the concentration of EDTA in the microinjection buffer can dramatically affect the viability of the embryos and thus, the frequency of obtaining transgenic mice. Use of microinjection buffers that lack EDTA result in reduced embryo survival and decreased integration efficiency (Brinster *et al.*, 1985) while use of microinjection buffers containing over 1 mM EDTA result in reduced embryo survival and severe toxicity. We recommend using a microinjection buffer composed of 5 mM Tris, pH 7.4 and 0.1 mM EDTA. Please see the next page for a recipe to prepare the microinjection buffer.

Most transgenic facilities develop their own guidelines and protocols to instruct users on how to prepare their transgene constructs for microinjection. We recommend that you consult your transgenic facility to obtain a protocol for preparing your DNA for microinjection and for a recipe for their preferred microinjection buffer.

Multiple steps must be performed to prepare your DNA fragment for microinjection (e.g. restriction digestion, agarose gel electrophoresis, electroelution, dialysis, CsCl gradient centrifugation, and dilution). You will lose a substantial amount of DNA from removal of the prokaryotic sequences, and you will likely lose some DNA at each purification step. Therefore, be sure that you start out with a sufficiently large amount of DNA when setting up your initial digestion. The higher the DNA concentration at the end of the purification process, the cleaner and easier it will be to inject after dilution. We recommend that you start with **at least** 100-300 μg of vector DNA for the initial digestion.

DNA Digestion and Gel Purification

General guidelines are provided below to isolate your DNA fragment for microinjection. Please see Hogan *et al.*, 1994 for details.

1. Use the appropriate restriction enzymes to digest your pBC1 construct such that the prokaryotic sequences are separated from the transgene construct (e.g. *Not* I and *Sal* I). Do not use *Not* I and *Sal* I if these restriction sites occur in your gene of interest.
2. Extract the DNA with phenol, then chloroform.
3. Separate the DNA fragments by electrophoresis on a TAE agarose gel containing 0.5 μg/ml ethidium bromide.
3. Use a longwave ultraviolet (UV) lamp to locate the band corresponding to the DNA fragment of interest and excise the band from the agarose gel.
4. Use any standard protocol of your choice to electroelute the DNA from the gel slice into a dialysis bag. Protocols may be found in most general references (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989). You may use an electroeluter, if available. Follow the manufacturer's instructions to electroelute the DNA.
5. Concentrate the eluted DNA using standard methods (i.e. S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-25) or CsCl gradient centrifugation).
6. Determine the concentration and purity of your isolated DNA fragment by reading the optical density at $OD_{260/280}$. You should have at least 50 μg of DNA at a concentration greater than 10 μg/ml before proceeding further. Proceed to purify your DNA fragment by CsCl gradient centrifugation (see the next page).

continued on next page

Preparation of DNA for Microinjection, continued

CsCl Gradient Centrifugation

Materials Needed

1X TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA)

Cesium Chloride

Ultracentrifuge tubes

Protocol

1. In a 50 ml conical centrifuge tube, bring your electroeluted DNA fragment (from the previous page) up in 10 ml of 1X TE buffer. Add 10 g CsCl to the DNA solution and mix gently to dissolve.
2. Load the DNA/CsCl solution into an ultracentrifuge tube. Seal the tube tightly with a heat sealer.
3. Centrifuge at 65,000 rpm for 6 hours or overnight in a vertical or near vertical rotor at room temperature.
4. Collect 0.5 ml fractions using a butterfly needle inserted approximately 1 cm from the bottom of the tube.
5. Run 3 μl of each fraction on an agarose gel containing 0.5 μg/ml ethidium bromide to identify the fractions containing your DNA fragment. Generally, fractions 7-12 contain the DNA, but this may vary depending on the size of the DNA fragment.
6. Combine the peak fractions into one tube.
7. Dialyze the DNA at +4°C against a 50-100X volume of microinjection buffer (see recipe below) for 24 hours. Change the microinjection buffer at least 3 times during dialysis.
8. After dialysis, run different dilutions of the DNA on an agarose gel with the proper standards to determine the concentration of your purified DNA. The concentration of DNA should be at least 5- to 10-fold higher than the concentration used for microinjection. Typically, the DNA concentration for microinjection is diluted to 200-400 molecules/picoliter (or 2-4 μg/ml for a 10 kb DNA fragment). We recommend that the concentration of your DNA fragment in your stock solution be at least 10 μg/ml.

Recipe for Microinjection Buffer

5 mM Tris, pH 7.4
0.1 mM EDTA

1. This solution can be prepared from the following common stock solutions. To prepare 1 liter, combine

1 M Tris, pH 7.4	5 ml
0.5 M EDTA	0.2 ml

2. Bring the volume up to 1000 ml with deionized water. Filter sterilize through a 0.45 μm filter.
3. Store tightly sealed at room temperature.

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Preparation of DNA for Microinjection, continued

Dilution of DNA into Microinjection Buffer

If you are sending your DNA for microinjection to a transgenic core facility or to a commercial facility to generate transgenic mice, you will often be asked to send concentrated DNA (see the previous page). Upon receipt of the DNA, the transgenic facility will dilute the DNA with microinjection buffer to the appropriate concentration immediately prior to microinjection.

If you are asked to provide DNA at a concentration suitable for microinjection, follow the protocol below to dilute your DNA to the appropriate concentration. Other protocols are suitable. Consult your transgenic facility to obtain a recipe for their preferred microinjection buffer.

Materials Needed

- Microinjection Buffer (5 mM Tris, pH 7.4, 0.1 mM EDTA) or preferred recipe
- 0.22 μm Millex-GV filter (Millipore, Catalog no. SLGV R25 LS)

Protocol

1. Dilute your concentrated stock of purified DNA to a final concentration of 200-400 molecules/picoliter with Microinjection Buffer (2-4 μg/ml for a 10 kb DNA fragment).
2. Filter the diluted DNA solution through a 0.2 μm filter.
3. Store at +4°C until needed for microinjection.

Generation of Transgenic Mice

Introduction

Once you have prepared your DNA for microinjection, you are ready to generate transgenic mice containing your construct of interest. As mentioned previously, we recommend that you collaborate with an experienced transgenic facility (either core or commercial) to generate your transgenic mice. General information about selection of a host strain and animal husbandry are provided below. For more detailed information, consult your transgenic facility.

Time Line

Please consult your transgenic facility to determine a suitable schedule for injection and generation of the first litter(s) of transgenic mice. In general, allow at least 6-7 weeks from the time of injection before obtaining the first set of mice to screen.

CD-1® Mouse Strain

We recommend using the CD-1® mouse as the host strain to generate transgenic mice. The CD-1® mouse strain is an outbred strain derived from a non-inbred stock of Swiss mice. The mice have an albino coat color, and are recommended for use in the generation of transgenic mice for this particular application because of the following reasons:

- fertilized eggs are relatively easy to microinject because pronuclei are large and easy to visualize
- female mice generally exhibit non-aggressive behavior during the milking process
- female mice are good mothers and are good milkers

CD-1® mice may be obtained from Charles River Laboratories. For more detailed information about the CD-1® strain, please contact Charles River Laboratories at:

Charles River Laboratories
251 Ballardvale St.
Wilmington, MA 01887
Tel: 1-800-LAB-RATS (1-800-522-7287)
Web Site: www.criver.com

Important

It is important that all mice be handled and housed in compliance with established Institutional Animal Guidelines. Please consult your transgenic and/or animal care facility for specific guidelines and recommendations to handle and care for your mice.

Care of Mice

In addition to following established protocols and guidelines to handle and care for your mice (see above), a number of other recommendations relating to the care of your mice for this particular application are listed below:

- Mice should be maintained on high-calorie, high-fat Purina mouse chow. This is particularly important for female mice during breeding as the high-calorie, high-fat diet will keep milk production high. Please consult your animal care facility for a supplier of the high-calorie, high-fat mouse chow.
- Once transgenic female mice have produced litters, it is critical that pups be kept healthy so that the mothers continue to lactate.

Other recommendations pertaining to the care of female transgenic mice during lactation are provided in the **Harvesting Milk** section (see pages 24-26).

CD-1® is a registered trademark of Charles River Laboratories

Identification of Transgenic Mice

Introduction

Once you have obtained the first litter(s) of mice from your transgenic core facility or commercial facility, you are ready to screen the mice to identify transgenic founders. Typically, 10% of mice generated are transgenic, although the frequency could vary depending on the nature of your insert. Due to variability in recombinant protein expression levels, we recommend that you obtain at least 8 confirmed transgenic female lines before proceeding to test for recombinant protein expression. To obtain at least 8 transgenic founder mice expressing the recombinant protein, we recommend the following:

- Inject approximately 300-500 eggs to obtain enough mice to screen for the transgene.
- Screen at least 100 mice to obtain a sufficient number of confirmed transgenic mice.
- Screen mice for the presence of the entire transgene to avoid obtaining mice which carry rearrangements or mutations within the transgene.

Please note that confirmed female transgenics can be bred and tested directly for protein production in the milk whereas male transgenics must first be mated to obtain female transgenic mice from the F1 generation. The F1 progeny must then be screened to identify the female transgenic mice.

Screening Mice

To screen mice for potential transgenic founders, you will need to perform a tail biopsy on each mouse. Genomic DNA will be prepared from each tail sample and subsequently screened by PCR analysis to detect the presence of the transgene. Tail samples that test positive in the PCR analysis can then be confirmed by restriction digestion and Southern blot analysis. **Note:** Tail samples should also be taken from wild-type mice to use as a negative control for the presence of the transgene.

Anesthetizing Mice

To perform tail biopsies, the mice will need to be anesthetized briefly. Many types of anesthetics (both inhalation and injectable) are suitable for use with mice. A protocol is provided on the next page to perform tail biopsies using an inhalation anesthetic, isoflurane, to anesthetize mice. Isoflurane (Aerrane®) may be obtained from Anaquest, Inc.

Anaquest, Inc.
110 Allen Road
Liberty Corner, NJ 07938
Tel: 908-647-9200
Fax: 908-604-7652

For more information about other available anesthetics, please consult your animal care facility.

Important

Mice should be handled in strict compliance with animal care guidelines during the anesthetization and tail biopsy procedure. Please consult your animal care facility for their recommended handling guidelines.

continued on next page

Identification of Transgenic Mice, continued

Tail Biopsies

A protocol is provided below to perform a tail biopsy on each mouse. Other protocols are suitable. For more details, please refer to *Manipulating the Mouse Embryo* (Hogan *et al.*, 1994).

1. Pipet 0.5 ml of isoflurane (see the previous page) into a 1 L beaker and cover the liquid with several paper towels.
2. Place the mouse inside the beaker and cover the beaker with foil. The mouse should lose consciousness within a minute or so. If the beaker is kept carefully covered, tail biopsies can be performed on up to 5 mice in succession. Do not keep mice under anesthetic for longer than 5 minutes.
3. Remove the anesthetized mouse from the beaker. Clip the mouse's ears for identification purposes. Using a sterile razor blade, cut 1 cm off the end of the tail. Minimal bleeding will occur, but no wound treatment is necessary as long as the razor blade is sterile.
4. Place the tail sample in a labeled microcentrifuge tube. Place the tube on ice.
5. Replace the mouse in the cage. The mouse should recover consciousness within a few minutes.
6. When you have finished collecting all of the tail samples, proceed directly to isolate genomic DNA from the mouse tails (see the next page) or store the samples at -70°C or in liquid nitrogen for later use.

Isolation of Genomic DNA

Use the Easy-DNA™ Kit supplied with the pBC1 Milk Expression Vector Kit to isolate genomic DNA from mouse tails for subsequent analysis. The Easy-DNA™ Kit contains enough reagents to isolate DNA from 150 samples. Approximately 125 µg of genomic DNA can generally be isolated from 1 cm of mouse tail. The Easy-DNA Kit™ is also available separately from Invitrogen (see page iv for ordering information).

continued on next page

Identification of Transgenic Mice, continued

Using the Easy-DNA™ Kit to Isolate DNA from Mouse Tails

Use the following protocol to isolate DNA from mouse tails using the Easy-DNA™ Kit. The procedure will take 2 days.

Day 1

Before starting, equilibrate a shaking water bath to 60°C. Thaw the Protein Degrader (if stored at -20°C) and keep on ice. If the solution is cloudy, warm at 37°C for 5 minutes until clear. If tail samples are frozen, warm at 37°C until thawed.

1. Into a sterile 50 ml capped centrifuge tube, mix the components in the volume listed below. Multiply each component by the number of samples (i.e. 100).

TE	320 µl
Solution A	20 µl
Solution B	10 µl
Protein Degrader (5 mg/ml)	5 µl

   Aliquot 355 µl of the mixture above into each mouse tail sample (fresh or frozen) and shake the microcentrifuge tubes at 60°C overnight (12-20 hours). Be sure to cap the tube tightly. **Note:** After overnight incubation, the mouse tail should be completely digested, with only hair visible in the solution. The solution will be cloudy and may be slightly colored depending on the color of the mouse tail.

Day 2

Before starting, equilibrate a 37°C heat block or water bath. Thaw RNase (if stored at -20°C) and keep on ice, and chill 100% and 80% ethanol in a -20°C freezer.

1. Add 300 µl Solution A and 120 µl Solution B to sample and vortex vigorously until solution is uniformly viscous (10 sec to 1 min).
2. Add 750 µl chloroform and vortex until the viscosity decreases and the mixture is homogeneous (10 sec to 1 min).
3. Centrifuge at maximum speed for 10 minutes at +4°C and transfer the upper aqueous phase to a fresh microcentrifuge tube.
4. If the upper phase is not clear, a second chloroform extraction is needed. Repeat steps 2 and 3. When upper phase is clear, proceed to the next step.
5. Add 1.0 ml of 100% ethanol (-20°C) to the clear upper phase. Vortex and incubate on ice for 30 minutes.
6. Centrifuge at maximum speed for 10 to 15 minutes at +4°C. Remove ethanol with a drawn-out pasteur pipette.
7. Add 500 µl 80% ethanol (-20°C) and mix by inverting the tube 3-5 times.
8. Centrifuge at maximum speed for 3 to 5 minutes at +4°C. Remove 80% ethanol with a drawn-out pasteur pipette.
9. Centrifuge the tube at maximum speed for 1-3 minutes at +4°C. Remove residual ethanol. Let air dry 5 minutes.
10. Resuspend the pellet in 49 µl TE and add 1 µl of 2 mg/ml RNase to a final concentration of 40 µg/ml. Incubate at 37°C for 30 minutes. DNA is ready for use. Store at +4°C. The typical yield is approximately 125 µg DNA for 1 cm of tail.

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Identification of Transgenic Mice, continued

Other DNA Isolation Protocols

Other protocols to isolate genomic DNA from mouse tails are suitable. An alternative protocol is included in the **Appendix** (see page 31). For more information, please refer to *Manipulating the Mouse Embryo* (Hogan *et al*., 1994).

PCR Analysis of Potential Founder Mice

Once you have isolated genomic DNA from the mouse tails, potential founder mice can easily be tested for the presence of the transgene using PCR analysis. Although PCR analysis is quick and easy, it is also subject to artifacts. Therefore, we recommend that all PCR analyses be performed with positive and negative controls in parallel. In addition, founder transgenic mice that test positive by PCR should be retested by Southern blot analysis. Southern blot analysis has the advantage of being less prone to false positives while also providing information about the structure, integrity, and copy number of the integrated transgene.

For PCR analysis, it will be necessary to design and synthesize two primers that will amplify a transgene-specific band of the appropriate size corresponding to your gene of interest. The PCR primers can be tested for specificity and sensitivity by performing test PCR on dilutions of transgene DNA that have been mixed with a standard amount of normal mouse genomic DNA. Alternatively, you may use the Forward and Reverse primers previously described (see page 12) for your PCR analysis.

Use the cycling parameters listed on page 13 or those optimized for your gene of interest to amplify DNA from the mouse tail genomic DNA samples. Amplified DNA may then be analyzed by agarose gel electrophoresis to identify potential transgenic mice.

Southern Blot Analysis

Southern blot analysis of potential founder mice should be planned carefully with regard to both the restriction digest and probe. Generally, a fragment of the transgene (100-500 bp) can easily be labeled using a standard random priming kit and used as the probe in the Southern blot. When choosing a restriction enzyme to digest the genomic DNA, we recommend choosing a restriction enzyme that cuts at known sites in the transgene and will yield a band of predictable size. Depending on your choice of probe and enzyme, you may also identify novel-sized “junction fragments” in addition to the predicted band. These junction fragment bands represent the ends of the integrated transgene array.

An estimate of transgene copy number can be obtained by including a range of standard amounts of the transgene mixed with normal mouse genomic DNA in parallel lanes on your Southern blot. For optimal results it is necessary to quantitate this DNA as accurately as possible. As a general rule, one copy of a typical 5-10 kb mouse gene is present in the mouse genome (6×10^9 bp) at approximately one part per million. Therefore, the amount of transgene construct used for the standards should cover the range from 1 to 100 pg. For a more detailed discussion about using Southern blot analysis to screen transgenic mice, please see Hogan *et al*., 1994.

Harvesting Milk

Introduction

Once you have identified at least 8 transgenic founder mice, you may proceed to breed the mice and harvest milk to test for expression of your recombinant protein. Remember that if any of your transgenic founders are male, you will need to mate the male founders. The resulting progeny will need to be screened to identify the F1 female transgenic mice. These F1 female transgenic mice may then be bred and tested for recombinant protein expression in the milk. The following section discusses factors to consider prior to harvesting milk and provides a protocol for harvesting milk.

Choosing Mice for Lactation

Once transgenic mice are confirmed by PCR and Southern blot, the mice must be prepared for lactation and milk testing. At four weeks of age, female transgenic mice can be mated. Once pups are born, the transgenic mother will begin lactation and milk can be tested.

General Notes on Milking Schedules

Once a transgenic female is producing milk, she can be tested for expression of the recombinant protein of interest. However, in developing a milking schedule, the health of the pups must be considered. In some cases, it may be advisable to have a foster mother on hand, but in most cases this will not be necessary as long as a limited milk harvesting schedule is followed. A number of recommendations to keep in mind when developing a milking schedule are provided below:

- Pups must remain healthy to maintain the mother's lactation cycle.
- In general, milking is best performed beginning on day 7 after the birth of the pups.
- The mother should not be milked on consecutive days to provide enough milk for the pups. Generally, mice may be milked every other day.
- Milk may be collected from the mother for approximately 3 weeks until the pups are weaned.

Please note that approximately 50-500 µl of milk may be harvested from a mouse at each milking. The mice do not need to be anesthetized during the milking process.

The Milking Apparatus

Before harvesting milk for the first time, you will need to assemble a milking apparatus to collect milk from the mouse. The milking apparatus uses a human breast pump that has been specially adapted to fit a mouse. A graphic and instructions to set up the milking apparatus are provided on the next page. To assemble the milking apparatus, you will need to have the following items on hand:

- Human breast pump (see the next page)
- 15 ml conical centrifuge tube
- Rubber stopper to fit the 15 ml centrifuge tube
- Two 18-gauge needles
- 12-15 inches of Tygon™ tubing to fit the breast pump and the hub of the 18-gauge needle
- 1.5 ml microcentrifuge tubes
- Tissues

Tygon™ is a trademark of Norton

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Harvesting Milk, continued

Human Breast Pump

We recommend using a human breast pump to harvest milk from the transgenic mice. We typically use the Medela Classic™ Electric Breastpump (Catalog no. 01501). For more information, please contact Medela directly at:

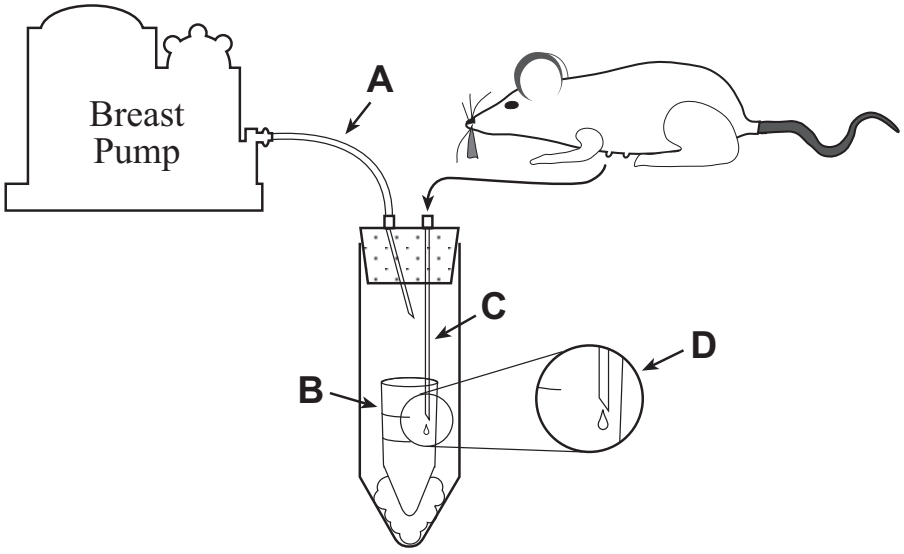
Tel: 1-800-435-8316 (U.S. and Canada)
Tel: +41-41-769 51 41 (Europe)
Web site: www.medela.com

Assembling the Milking Apparatus

To assemble the milking apparatus, follow the instructions listed below. A diagram of the milking apparatus is provided below for your convenience.

1. Attach the Tygon™ tubing to the breast pump.
2. Connect the tubing from the pump to the hub of an 18-gauge needle inserted diagonally through the rubber stopper (see **A** below).
3. Place tissues into the bottom of the 15 ml conical centrifuge tube such that the bottom of the tube is cushioned.
4. Remove the cap from the 1.5 ml microcentrifuge tube and insert the microcentrifuge tube into the 15 ml conical centrifuge tube such that it stands vertically and stably within the 15 ml tube (see **B** below).
5. Insert a second 18-gauge needle vertically through the rubber stopper. The tip of the needle should lie within the microcentrifuge tube when the rubber stopper is placed on the 15 ml conical tube (see **C** below).

   Note: The needle should be turned and positioned so that the beveled portion of the needle faces towards the center of the microcentrifuge tube (see **D** below).
6. Place the rubber stopper in the 15 ml conical centrifuge tube and check to see that suction is generated through the hub of the needle when the breast pump is turned on. Proceed to milk the mouse (see the next page).
7. After the mouse has been milked, the microcentrifuge tube (see **B** below) containing the expressed milk should be removed from the 15 ml conical tube and replaced with a fresh microcentrifuge tube.



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Harvesting Milk, continued

Protocol for Milking

Beginning on day 7 after the delivery of the pups, transgenic mothers can be milked to begin expression analysis experiments. Milk may be harvested every other day from the mice. We recommend that milking take place in a quiet room as nervous or stressed mice are harder to milk. **Note:** Remember to harvest milk from a wild-type female mouse to use as a negative control for recombinant protein expression.

1. The mother should be isolated from pups 1 hour prior to the planned milking time to allow milk to accumulate.
2. Just prior to milking (approximately 1 minute) the mother should be injected intra-peritoneally with 5 i.u. of oxytocin (Sigma, Catalog no. O2882) using a 25-gauge needle. Oxytocin induces expression of the milk. The total volume of oxytocin injected should be approximately 0.2 cc. The hormone should take effect within 1 to 5 minutes after injection.
3. Turn on the breast pump for the assembled milking apparatus.
4. To milk the mouse, hold it by the base of the tail so that the mouse is facing away from you. Let the mouse grasp the edge of the cage with its front paws. The mouse need not be otherwise restricted or confined. Gently lift the mouse's hindquarters to reveal the teats. Lift the 15 ml conical tube to the mouse and allow the suction to draw the mouse teat into the needle hub (see diagram on the previous page). Milk each teat until the milk supply is exhausted. Generally, approximately 50-500 µl of milk can be obtained from the mouse in a single milking.
5. Store the milk on ice for immediate analysis or at –80°C for later analysis.

Purification of Proteins from Milk

Introduction

The purpose of the pBC1 Milk Expression Kit is to facilitate recombinant protein production in the milk of mice for feasibility studies. For initial testing of expression, it is generally not necessary to purify the protein away from milk, however, a greater degree of purification may be required depending on the nature of your recombinant protein of interest and the nature of your analysis. Several approaches for protein purification from milk are discussed in the following section.

For a general reference on biochemical purifications, we recommend that you refer to *Methods in Enzymology*, volume 182 (Deutscher, 1990) or *Current Protocols in Protein Science* (Coligan *et al.*, 1995). Some general strategies for purifying proteins from milk are reviewed in Young *et al.*, 1997 and Ziomek, 1998. For large scale recombinant protein purification, collaboration with an experienced protein biochemist is advisable.

Initial Expression Testing

Initial testing for expression of recombinant protein in mouse milk generally involves dilution of the milk and analysis by SDS-polyacrylamide gel electrophoresis. General guidelines are provided below to prepare samples for analysis. Either fresh or frozen milk may be tested. If frozen milk is used, thaw the milk before proceeding. Remember to include a sample of milk from a wild-type mouse as a negative control for expression. Purified recombinant protein may be used as a positive control.

1. Dilute the milk 20-fold in sterile water.
2. Remove 10 µl of the diluted milk and dilute 1:1 in 2X SDS-PAGE sample buffer.
3. Incubate the sample at 65°C for 10 minutes.
4. Load 4 µl of sample on an SDS-polyacrylamide gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your recombinant protein.
5. Proteins may be visualized by Coomassie-blue staining or western blot analysis.

Western Blot Analysis

If you wish to perform western blot analysis to assay for your recombinant protein, you will need to have an antibody to your recombinant protein. We recommend that you use a monoclonal antibody to detect your recombinant protein as monoclonal antibodies are less likely to cross-react with host proteins.

Strategies for Purification

The exact strategy for further purification of your recombinant protein from milk will depend on the chemistry of your protein. Several recombinant proteins have been purified using immunoabsorption with a monoclonal antibody (Denman *et al.*, 1991; Hansson *et al.*, 1994). In other cases, a strategy relying on conventional chromatography such as cation/anion exchange have been used (Edmunds *et al.*, 1998). As an example of a purification strategy, the general steps used by Genzyme Transgenics Corporation to purify rhAntithrombin III to 99.999% purity from transgenic goat milk is shown in the **Appendix** (see page 32). To develop a purification scheme for your recombinant protein, we recommend collaboration with an experienced protein biochemist.

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Purification of Proteins from Milk, continued

Removal of Fats

Generally, the first step to purify a protein from milk usually includes a clarification step that removes most of the fat and lipid from the milk. Fats can be removed from the milk via microfiltration or by centrifugation of the diluted milk at 8000 x g for 5 minutes. The resulting layer of fat is skimmed off the top (Hansson *et al.*, 1994). Further purification steps may vary depending on the nature of your recombinant protein.

Scale Up into Larger Animals

Once you have completed feasibility studies in transgenic mice, you may use the pBC1 vector to express your recombinant protein in larger animals. Scale up into larger animals requires that you enter into a commercial agreement with Genzyme Transgenics Corporation. For more information, please contact:

Commercial Development
Genzyme Transgenics Corporation
5 Mountain Road
Framingham, MA 01701-9322
Tel: 508-872-8400
Fax: 508-370-3797

Appendix

Proteins Expressed in Transgenic Animal Milk

Proteins Expressed in Milk

The pBC1 milk expression vector has been used to express a number of recombinant proteins and antibodies in transgenic animals. The following table lists some of the proteins and antibodies which have been expressed from pBC1 in the milk of transgenic mice and goats as well as information about the expression levels achieved in each transgenic model. For more information, please refer to the Genzyme Transgenics Corporation Web site (www.genzyme.com/transgenics) or contact Genzyme Transgenics Corporation (see page 35).

Protein	Animal	Expression Level (g/L)	Reference
Recombinant Proteins			
Human long-acting tissue plasminogen activator (tPA)	Mouse Goat	6 6	(Ebert *et al.*, 1994; Young *et al.*, 1997; Ziomek, 1998)
Antithrombin III	Mouse Goat	10 14	(Edmunds *et al.*, 1998; Young *et al.*, 1997; Ziomek, 1998)
α1-Proteinase Inhibitor	Mouse Goat	35 20	(Young *et al.*, 1997; Ziomek, 1998)
Human Serum Albumin	Mouse	35	(Young *et al.*, 1997)
Soluble CD4 HIV Receptor	Mouse	8	(Young *et al.*, 1997)
Antibodies			
Anti-cancer Monoclonal Antibody (MAb)	Mouse Goat	10 10	(Young *et al.*, 1997; Ziomek, 1998)
Anti-Lewis Y, BR96 MAb	Mouse Goat	4 14	(Young *et al.*, 1997; Ziomek, 1998)
Human Transferrin Receptor MAb	Mouse	2	(Young *et al.*, 1997)
Single-chain Antibody	Mouse	1	(Young *et al.*, 1997)

Colony Hybridization

Introduction

A protocol for using colony hybridization to screen for *E. coli* transformants containing your insert of interest is provided below. Other protocols are suitable. For additional details, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989). Remember to include a plate containing pBC1 vector alone as a negative control for hybridization. A diluted sample of insert alone may be spotted onto a filter to serve as a positive control.

Colony Hybridization Protocol

Replica-plating Colonies to Filter

1. With a soft lead pencil, label dry filters to be used (DuPont Plaque Screen Filters, Catalog no. NEF978A). Wet filter with water and sandwich between dry Whatman 3MM paper. Wrap the stack of filters in aluminum foil and autoclave to sterilize.
2. Plate the transformation on LB agar plates containing 50 µg/ml ampicillin. Lay a Plaque Screen filter on top of the agar and incubate the LB plate overnight at 37°C.
3. Prepare 4 glass trays for cell lysis and binding. Cut 4 pieces of Whatman 3MM paper to the size and shape of a glass tray. Place each piece of Whatman paper in a separate glass tray and saturate each tray with one of the following solutions listed below. Pour off excess liquid.

Tray	Solution	Purpose
1	10% SDS	Reduce background
2	0.5 N NaOH 1.5 M NaCl	Denaturing solution
3	1.5 M NaCl 0.5 M Tris, pH 7.4	Neutralizing solution
4	0.3 M NaCl 30 mM Sodium citrate	Wash solution

4. Using blunt-ended forceps, peel the Plaque Screen filter from the plate and place in the glass tray prepared with 10% SDS. Be sure to place the filter colony side up. Incubate the filter in SDS for 3 minutes.
5. Transfer the filter sequentially to the second tray for 5 minutes. Transfer the filter to the third tray for 5 minutes. Finally, transfer the filter to the fourth tray for 5 minutes. Make sure that the filters are always placed in each tray colony side up.
6. Lay the filters, labeled side up on a sheet of 3MM paper. Allow them to dry fully at room temperature.
7. Sandwich the filters between sheets of dry 3MM paper and fix by baking for 1 hour in an 80°C vacuum oven.
8. The filters are now ready for hybridization with a labeled probe.

Probes to Use for Hybridization

You may use a labeled oligonucleotide or a nick-translated DNA fragment from your insert as a probe to detect those transformants containing your insert of interest. For more information about labeling your oligonucleotide or DNA fragment, please refer to *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Isolation of Genomic DNA

Introduction

A protocol for isolation of genomic DNA from mouse tails is provided below for your convenience. Other protocols are suitable. For more information and other protocols, please refer to Hogan *et al.*, 1994. Please note that the Easy-DNA™ Kit is supplied with the pBC1 Milk Expression System for easy isolation of genomic DNA from mouse tails (see page 22 for a protocol and page iv for ordering information).

Isolating High Molecular Weight DNA from Mouse Tails

Materials:

1 cm of mouse tail

1.5 ml microcentrifuge tubes

50 mM Tris (pH 8.0), 100 mM EDTA, 0.5% SDS

Proteinase K (10 mg/ml in water)

Phenol, equilibrated with Tris-HCl, pH 8.0

Phenol: Chloroform (1:1, v/v)

3 M Sodium acetate, pH 6.0

70% and 100% ethanol at room temperature

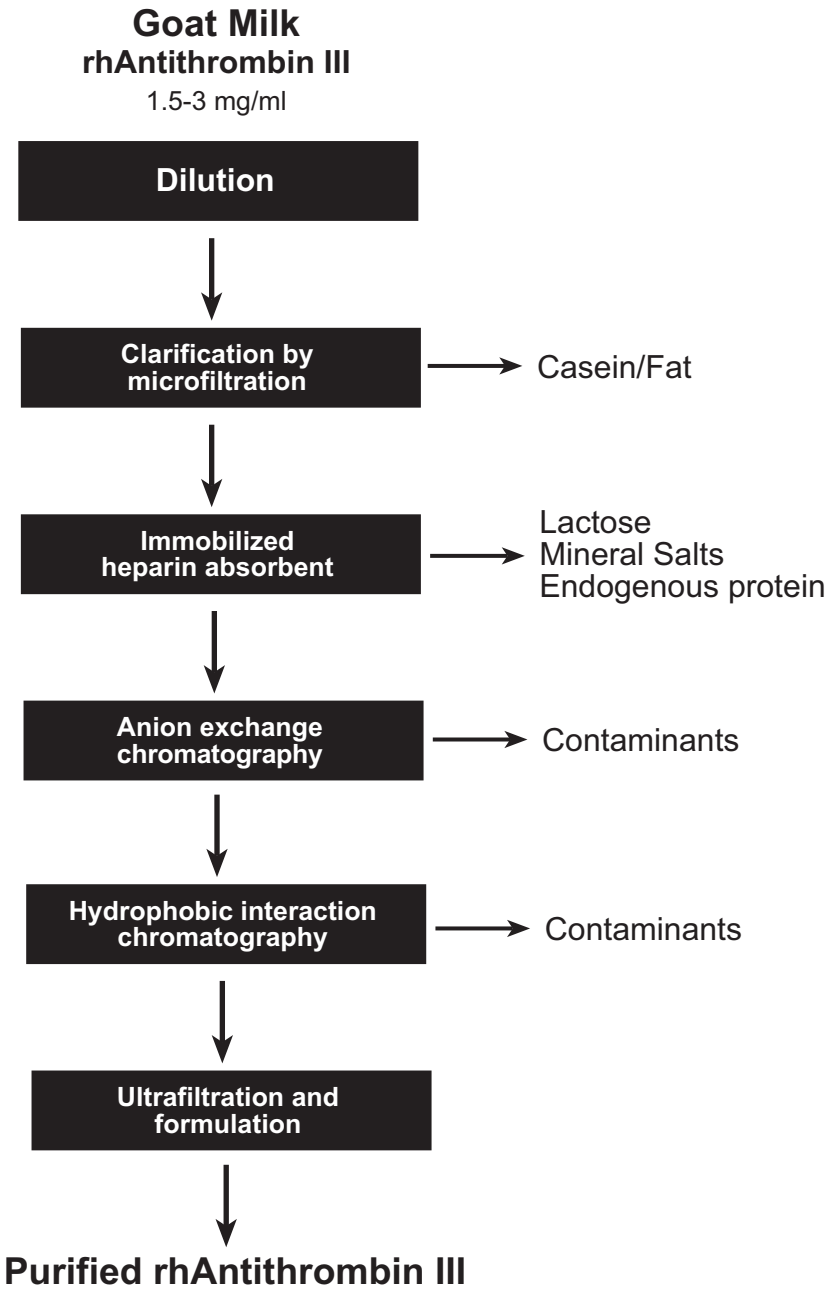
1X TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA)

1. Place the mouse tail sample in a microcentrifuge tube and add 0.5 ml of 50 mM Tris, pH 8.0, 100 mM EDTA, 0.5% SDS. Add 25 µl of a 10 mg/ml stock of Proteinase K. Incubate overnight at 55°C in a shaking water bath.
2. Add 0.5 ml of equilibrated phenol to the digested tail and shake vigorously for 3 minutes. **Note:** Do not vortex. Vortexing will shear the genomic DNA.
3. Centrifuge the tube for 3 minutes at top speed to separate the organic and aqueous phases. Transfer the aqueous (top) phase to a fresh tube.
4. Re-extract the aqueous phase with 0.5 ml of phenol:choloroform. Shake vigorously for 3 minutes and centrifuge at top speed for 3 minutes.
5. Remove aqueous (top) phase to a fresh tube.
6. Precipitate DNA by adding 50 µl of 3 M sodium acetate (pH 6.0) and 0.5 ml of 100% ethanol to the tube. Invert the tube gently to mix. DNA should immediately be visible as a stringy precipitate. Please note that using sodium acetate with lower pH will cause EDTA to precipitate.
7. To pellet the DNA, centrifuge at top speed for 30 seconds. Remove ethanol with a pipette.
8. Wash the pellet with 1 ml of 70% ethanol.
9. Centrifuge at top speed in the microcentrifuge for 1 minute. Remove the ethanol.
10. Resuspend the DNA pellet in 1X TE buffer.

Sample Recombinant Protein Purification Strategy

Purification of rhAntithrombin III

The strategy used to purify recombinant Antithrombin III from transgenic goat milk is provided below as an example of schemes available for purifying proteins from milk. Such purification schemes may be modified according to the nature of your recombinant protein of interest. In this case, the overall yield of recombinant Antithrombin III obtained was 53% and the purity achieved was 99.999% (Edmunds *et al.*, 1998). For more information, please contact Genzyme Transgenics Corporation (see page 35).



Technical Service

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Product Qualification

Introduction

The following criteria are used to qualify the components of the pBC1 Milk Expression Vector Kit.

Vector

The pBC1 vector is qualified by restriction enzyme digestion with specific restriction enzymes as listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel (see below).

Restriction Enzyme	Expected Results (bp)
Afl I	1207, 3730, 4215, 12476
*Hin*d III	1207, 3319, 4450, 4503, 8149
Sap I	1202, 1207, 4717, 5392, 9110
Sph I	720, 4733, 7266, 8909

Easy-DNA™ Kit

Each kit component is sterile, free of nuclease contamination, and is lot qualified for optimum performance. Greater than 10 μg of high molecular weight DNA must be isolated from 10^6 Sf9 insect cells.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Brinster, H. L., Chen, N. Y., Trumbauer, M. E., Yagle, M. K., and Palmiter, R. D. (1985). Factors Affecting the Efficiency of Introducing Foreign DNA into Mice by Microinjecting Eggs. Proc. Natl. Acad. Sci. USA *82*, 4438-4442.

Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. (1988). Introns Increase Transcriptional Efficiency in Transgenic Mice. Proc. Natl. Acad. Sci. USA *85*, 836-840.

Chada, K., Magram, J., Raphael, K., Radice, G., Lacy, E., and Constantini, F. (1985). Specific Expression of a Foreign Beta-globin Gene in Erythroid Cells of Transgenic Mice. Nature *314*, 377-380.

Choi, T., Huang, M., Gorman, C., and Jaenisch, R. (1991). A Generic Intron Increases Gene Expression in Transgenic Mice. Mol. Cell. Biol. *11*, 3070-3074.

Chung, J. H., Bell, A. C., and Felsenfeld, G. (1997). Characterization of the Chicken β-globin Insulator. Proc. Natl. Acad. Sci. USA *94*, 575-580.

Chung, J. H., Whiteley, M., and Felsenfeld, G. (1993). A 5´ Element of the Chicken β-Globin Domain Serves as an Insulator in Human Erythroid Cells and Protects against Position Effect in Drosophila. Cell *74*, 505-514.

Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. (1995). Current Protocols in Protein Science (New York: John Wiley).

Denman, J., Hayes, M., O'Day, C., Edmunds, T., Bartlett, C., Hirani, S., Ebert, K. M., Gordon, K., and McPherson, J. M. (1991). Transgenic Expression of a Variant of Human Tissue-type Plasminogen Activator in Goat Milk: Purification and Characterization of the Recombinant Enzyme. BioTechnology *9*, 839-843.

Deutscher, M. P. (1990) Guide to Protein Purification. In *Methods in Enzymology*, Vol. 182. (J. N. Abelson and M. I. Simon, eds.) Academic Press, San Diego, CA.

Ebert, K. M., DiTullio, P., Barry, C. A., Schindler, J. E., Ayres, S. L., Smith, T. E., Pellerin, L. J., Meade, H. M., Denman, J., and Roberts, B. (1994). Induction of Human Tissue Plasminogen Activator in the Mammary Gland of Transgenic Goats. BioTechnology *12*, 699-702.

Edmunds, d., Patten, S. M. V., Pollock, J., Hanson, E., Bernasconi, R., Higgins, E., Manavalan, P., Ziomek, C., Meade, H., McPherson, J. M., and Cole, E. S. (1998). Transgenically Produced Human Antithrombin: Structural and Functional Comparison to Human Plasma-Derived Antithrombin. Blood *91*, 4561-4571.

Hansson, L., Edlund, M., Edlund, A., Johansson, T., Marklund, S. L., Fromm, S., Strömqvist, M., and Törnell, J. (1994). Expression and Characterization of Biologically Active Human Extracellular Superoxide Dismutase in Milk of Transgenic Mice. J. Biol. Chem. *269*, 5358-5363.

Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994). Manipulating the Mouse Embryo, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Hohn, B., and Collins, J. (1980). A Small Cosmid for Efficient Cloning of Large DNA Fragments. Gene *11*, 291-298.

Kozak, M. (1987). An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nuc. Acids Res. *15*, 8125-8148.

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References, continued

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biol. *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Krumlauf, R., Hammer, R. E., Tilghman, S. M., and Brinster, R. L. (1985). Developmental Regulation of Alpha-fetoprotein in Transgenic Mice. Mol. Cell. Biol. *5*, 1639-1648.

Maga, E. A., and Murray, J. D. (1995). Mammary Gland Expression of Transgenes and the Potential for Altering the Properties of Milk. Biotechnology *13*, 1452-1457.

Meade, H. M., Echelard, Y., Ziomek, C. A., Young, M. W., Harvey, M., Cole, E. S., Groet, S., Smith, T. E., and Curling, J. M. (1999) Expression of Recombinant Proteins in the Milk of Transgenic Animals. In *Gene Expression Systems: Using Nature for the Art of Expression*, J. M. Fernandez and J. P. Hoeffler, eds. (San Diego, CA: Academic Press), pp. 399-427.

Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Allen, D. D., and Brinster, R. L. (1991). Heterologous Introns Can Enhance Expression of Transgenes in Mice. Proc. Natl. Acad. Sci. USA *88*, 478-482.

Persuy, M. A., Legrain, S., Printz, C., Stinnakre, M. G., Lepourry, L., Brignon, G., and Mercier, J. C. (1995). High-level, Stage- and Mammary-tissue-specific Expression of a Caprine κ-casein-encoding Minigene Driven by a β-casein Promoter in Transgenic Mice. Gene *165*, 291-296.

Persuy, M. A., Stinnakre, M. G., Printz, C., Mahe, M. F., and Mercier, J. C. (1992). High Expression of the Caprine β-casein Gene in Transgenic Mice. Eur. J. Biochem. *205*, 887-893.

Roberts, B., DiTullio, P., Vitale, J., Hehir, K., and Gordon, K. (1992). Cloning of the Goat β-casein-encoding Gene and Expression in Transgenic Mice. Gene *121*, 255-262.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Strouboulis, J., Dillon, N., and Grosveld, F. (1992). Developmental Regulation of a Complete 70-kb Human Beta-globin Locus in Transgenic Mice. Genes Dev. *6*, 1857-1864.

Talbot, D., Collis, P., Antoniou, M., Vida, M., Grosveld, F., and Greaves, D. R. (1989). A Dominant Control Region from the Human β-globin Locus Conferring Integration Site-Independent Gene Expression. Nature *338*, 352-355.

Townes, T. M., Lingrel, J. B., Brinster, R. L., and Palmiter, R. D. (1985). Erythroid Specific Expression of Human Beta-globin Genes in Transgenic Mice. EMBO J. *4*, 1715-1723.

Young, M. W., Okita, W. B., Brown, E. M., and Curling, J. M. (1997). Production of Biopharmaceutical Proteins in the Milk of Transgenic Dairy Animals. BioPharm *10*, 34-38.

Ziomek, C. A. (1998). Commercialization of Proteins Produced in the Mammary Gland. Theriogenology *49*, 139-144.

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